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(54) Title: EMBRYONIC STEM CELL CULTURE COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract: The present invention provides compositions and methods for the culture and maintenance of undifferentiated, pluripotent mammalian stem cells. More particularly, the present invention provides a defined media useful in the absence of a feeder layer and in the absence of serum or serum replacement, that comprises an activator of IGF-IR, an activator of an FGF receptor, serum albumin, transferrin, and optionally, a member of the TGF- $\beta$  family.

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**EMBRYONIC STEM CELL CULTURE COMPOSITIONS AND  
METHODS OF USE THEREOF**

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[0001] This invention was made, at least in part, with funding from the National Institutes of Health (Grant Number 9 R24 RR021313-04). Accordingly, the 10 United States Government has certain rights in this invention.

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

[0002] The present invention generally relates to compositions and methods 15 for culturing pluripotent stem cells, the cells created by these methods, and their uses thereof. Particularly, the invention relates to a defined medium for culturing pluripotent stem cells in the absence of a feeder cell layer, and in the absence of serum or serum replacement.

20 **Background Art**

[0003] Embryonic stem (ES) cells represent a powerful model system for the investigation of mechanisms underlying pluripotent cell biology and differentiation within the early embryo, as well as providing opportunities for genetic manipulation of mammals and resultant commercial, medical and agricultural applications. 25 Furthermore, appropriate proliferation and differentiation of ES cells can be used to generate an unlimited source of cells suited to transplantation for treatment of diseases that result from cell damage or dysfunction. Other pluripotent cells and cell lines including early primitive ectoderm-like (EPL) cells as described in International Patent Application WO 99/53021, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells (EG cells), teratocarcinoma cells (EC cells), and pluripotent cells derived by dedifferentiation or by nuclear transfer will 30 share some or all of these properties and applications.

[0004] The successful isolation, long-term clonal maintenance, genetic manipulation, and germ-line transmission of pluripotent cells has generally been

difficult, and the reasons for this are unknown. PCT Publication No. WO 97/32033 and U.S. Patent No. 5,453,357 describe pluripotent cells including cells from species other than rodents. Human ES cells have been described in International Patent Application Publication No. WO 00/27995, and in U.S. Patent No. 6,200,806, and 5 human EG cells have been described in PCT Publication No. WO 98/43679.

10 [0005] The biochemical mechanisms regulating ES cell pluripotency and differentiation are very poorly understood. However, the limited empirical data available (and much anecdotal evidence) suggests that the continued maintenance of pluripotent ES cells under *in vitro* culture conditions is dependent upon the presence of cytokines and growth factors present in the extracellular milieu.

15 [0006] Other groups have made progress toward developing feeder-free conditions for hESCs (Xu *et al.*, 2005, *Stem Cells*, 23:315-323; Xu *et al.*, 2005, *Nature Methods*, 2:185-189; Beattie *et al.*, 2005, *Stem Cells*, 23:489-495; Klimanskaya *et al.*, 2005, *Lancet* 365:1636-1641; Amit *et al.*, 2004, *Biol. Reprod.* 70:837-845; James *et* 20 *al.*, 2005, *Development*, 132:1279-1282), however, all research to date has used knockout serum replacer (KSR; Invitrogen Corp.) in the media. While the formulation of KSR has not been published, it is apparent from PCT Publication No. WO/98/30679 that KSR contains high levels of insulin, as well as other possible sources of growth factors, such as potentially found in Albumax® (Invitrogen Corp.). In addition, the extracellular matrix may also have been a source of matrix-associated growth factors.

25 [0007] An important development in the progression of hESC research toward these goals will be the elucidation of media and cell culture conditions that are compatible with the expected regulatory guidelines governing clinical safety and efficacy. While the best outcome would be the availability of chemically defined media for hESCs, components that are not chemically defined would be acceptable if they were produced to GMP standard. There is a need, therefore, to identify methods and compositions for the culture and stabilization of a population of pluripotent stem cells, wherein the culture compositions are defined and/or produced to GMP standard.

### 30 SUMMARY OF THE INVENTION

[0008] It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art. One embodiment of the present invention relates to a novel, defined composition comprising a basal salt nutrient solution, an activator of the IGF-1 receptor (IGF-1R),

serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free. The invention also encompasses a composition for culturing a pluripotent mammalian embryonic stem cell, comprising an extracellular matrix, and a medium comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the medium is essentially serum free, wherein a pluripotent mammalian stem cell remains undifferentiated for greater than approximately 5 passages in culture.

5 [0009] Also contemplated is a cell composition comprising a pluripotent mammalian embryonic stem cell proliferating on an extracellular matrix in the presence of a defined medium, wherein the cell composition is essentially free of feeder cells, and wherein the defined medium comprises a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family.

10 [0010] The invention is further directed to a method of culturing a pluripotent mammalian embryonic stem cell comprising (a) providing a pluripotent mammalian embryonic stem cell; (b) plating the cell on an extracellular matrix; and (c) contacting the cell with a defined medium that is essentially serum free comprising an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a 15 member of the TGF- $\beta$  family, wherein the stem cell proliferates in culture and remains undifferentiated essentially in the absence of serum or serum replacement in the medium. In one embodiment, the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than 5 passages in culture. In another embodiment, the pluripotent mammalian embryonic stem cell proliferates and 20 remains undifferentiated for greater than one month in culture. In another embodiment, the contact of the cell with the defined medium is in the absence of a feeder layer. In another embodiment, the pluripotent mammalian embryonic stem cell is cultured on feeder cells prior to plating on the extracellular matrix.

25 [0011] In particular embodiments of the invention, the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2 and BMP4. In a further embodiment, the member of the TGF- $\beta$  family is Activin A. It is contemplated that the activator of IGF-1R is selected from the group 30 consisting of insulin and an insulin-like growth factor. In one embodiment, the

insulin-like growth factor is IGF-1 or IGF-2. In one embodiment, the insulin-like growth factor is IGF-1, which can be LongR<sup>3</sup>IGF-1. In one embodiment, the activator of an FGF receptor is FGF2. It is also contemplated that the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin. In a 5 further embodiment, the medium is a non-conditioned medium.

[0012] The mammalian cell population used in the invention is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell. In one embodiment, the mammalian cell population comprises a human embryonic stem cell.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figures 1A-D show photomicrographs of BG01v colonies grown in conditioned medium (CM) comprising FGF2 and BSA (Panel A); FGF2, insulin, and Activin A (Panel B); FGF, insulin, and BSA (Panel C); and FGF, LongR<sup>3</sup>-IGF-1, and 15 BSA (Panel D).

[0014] Figures 2A-G show photomicrographs of BG01v colonies grown in medium comprising FGF2 and insulin (Panel A); FGF and LongR<sup>3</sup>-IGF-1 (Panel B); FGF2 (Panel C); insulin (Panel D); LongR<sup>3</sup>-IGF-1 (Panel E); FGF2, and Insulin (Panel F); and FGF2 and LongR<sup>3</sup>-IGF-1 (Panel G). Panels A and B are in the presence of 20 CM, while Panels C-G are in the presence of non-conditioned medium.

[0015] Figures 3A-D show photomicrographs of BG01v colonies grown in non-conditioned medium comprising FGF2 and LongR<sup>3</sup>-IGF-1 (Panels A and B); and FGF2, LongR<sup>3</sup>-IGF-1, and Activin A (Panels C and D).

[0016] Figures 4A-E show the characteristics of a p2 culture. Using RT-PCR, 25 Panel A shows that the cultures expressed multiple markers of pluripotency, including ABCG2, DPPA5, REX1, UTF1, NANOG, OCT4, SOX2, CRIPTO, and TERT. Panel B shows a photomicrograph of a DAPI stained colony. Panels C and D show immunostainings of the same colony as Panel B, demonstrating the uniform expression of Oct4 (Panel C), and SSEA-4 (Panel D). The colonies were also uniformly positive 30 for alkaline phosphatase activity.

**DETAILED DESCRIPTION OF THE INVENTION**

[0017] Applicants have demonstrated that culturing pluripotent mammalian cells in a defined medium in the absence of a feeder layer, serum, and serum replacement allows for the maintenance of said cells in an undifferentiated state.

5 [0018] One embodiment of the present invention relates to a novel, defined composition comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free. The invention also encompasses a composition for culturing a pluripotent mammalian embryonic stem cell, comprising an extracellular matrix, and a medium comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the medium is essentially serum free, wherein a pluripotent mammalian stem cell remains undifferentiated for greater than approximately 5 passages in culture. In one 10 embodiment, the pluripotent mammalian stem cell remains undifferentiated for greater than approximately 1 month in culture.

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[0019] Also contemplated is a cell composition comprising a pluripotent mammalian embryonic stem cell proliferating on an extracellular matrix in the presence of a defined medium, wherein the cell composition is essentially free of feeder cells, and wherein the defined medium comprises a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family.

20 [0020] The invention is further directed to a method of culturing a pluripotent mammalian embryonic stem cell comprising (a) providing a pluripotent mammalian embryonic stem cell; (b) plating the cell on an extracellular matrix; and (c) contacting the cell with a defined medium that is essentially serum free comprising an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the stem cell proliferates in culture and remains undifferentiated essentially in the absence of serum or serum replacement in the 25 medium. In one embodiment, the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than 5 passages in culture. In a further embodiment, the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than 1 month in culture. In another embodiment, 30

the contact of the cell with the defined medium is in the absence of a feeder layer. In another embodiment, the pluripotent mammalian embryonic stem cell is cultured on feeder cells prior to plating on the extracellular matrix.

[0021] In particular embodiments of the invention, the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4. In a further embodiment, the member of the TGF- $\beta$  family is Activin A. It is contemplated that the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor. In one embodiment, the insulin-like growth factor is IGF-1 or IGF-2. In a further embodiment, the insulin-like growth factor is IGF-1, which can be LongR<sup>3</sup>IGF-1. In one embodiment, the activator of an FGF receptor is FGF2. It is contemplated that FGF2 is initially present at a concentration of approximately 0.1 ng/ml to approximately 100 ng/ml, approximately 0.5 ng/ml to approximately 50 ng/ml, approximately 1 ng/ml to approximately 25 ng/ml, approximately 1 ng/ml to approximately 12 ng/ml, or is initially present at a concentration of approximately 8 ng/ml.

[0022] It is also contemplated that the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin. In a further embodiment, the medium is a non-conditioned medium.

[0023] The mammalian cell population used in the invention is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell. In one embodiment, the mammalian cell population comprises a human embryonic stem cell.

[0024] Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art. In addition to the definitions of terms provided below, definitions of common terms in molecular biology may also be found in Rieger *et al.*, 1991, *Glossary of genetics: classical and molecular*, 5th Ed., Berlin: Springer-Verlag; and in *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement). It is to be understood that as used in the specification and in the claims, “a” or “an” can mean one or more, depending upon the context in which it is used. Thus, for example, reference to “a cell” can mean that at least one cell can be utilized.

[0025] The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present compositions and methods are disclosed and described, it is to be understood that this invention is not limited to specific nucleic acids, specific polypeptides, specific cell types, specific host cells, specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art.

[0026] Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases, and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.*, 1989, *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis *et al.*, 1982, *Molecular Cloning*, Cold Spring Harbor Laboratory, Plainview, New York; Wu (Ed.), 1993, *Meth. Enzymol.* 218, Part I; Wu (Ed.), 1979, *Meth. Enzymol.* 68; Wu *et al.*, (Eds.), 1983, *Meth. Enzymol.* 100 and 101; Grossman and Moldave (Eds.), 1980, *Meth. Enzymol.* 65; Miller (ed.), 1972, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose, 1981, *Principles of Gene Manipulation*, University of California Press, Berkeley; Schleif and Wensink, 1982, *Practical Methods in Molecular Biology*; Glover (Ed.), 1985, *DNA Cloning* Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (Eds.), 1985, *Nucleic Acid Hybridization*, IRL Press, Oxford, UK; and Setlow and Hollaender, 1979, *Genetic Engineering: Principles and Methods*, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

[0027] Human pluripotent cells offer unique opportunities for investigating early stages of human development as well as for therapeutic intervention in several disease states, such as diabetes mellitus and Parkinson's disease. For example, the use of insulin-producing  $\beta$ -cells derived from hESCs would offer a vast improvement over current cell therapy procedures that utilize cells from donor pancreases. Currently cell therapy treatments for diabetes mellitus, which utilize cells from donor pancreases, are limited by the scarcity of high quality islet cells needed for transplant. Cell therapy for a single Type I diabetic patient requires a transplant of approximately  $8 \times 10^8$

pancreatic islet cells (Shapiro *et al.*, 2000, *N. Engl. J. Med.*, 343:230-238; Shapiro *et al.*, 2001a, *Best Pract. Res. Clin. Endocrinol. Metab.*, 15:241-264; Shapiro *et al.*, 2001b, *Bmj.*, 322:861). As such, at least two healthy donor organs are required for to obtain sufficient islet cells for a successful transplant. HESCs offer a source of starting material from which to develop substantial quantities of high quality differentiated cells for human cell therapies.

5 **[0028]** As used herein, a basal salt nutrient solution refers to a mixture of salts that provide cells with water and certain bulk inorganic ions essential for normal cell metabolism, maintain intra- and extra-cellular osmotic balance, provide a carbohydrate 10 as an energy source, and provide a buffering system to maintain the medium within the physiological pH range. Non-limiting examples of basal salt nutrient solutions include Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, Ham's F-10, Ham's F-12,  $\alpha$ -Minimal Essential Medium ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-15 MEM), and Iscove's Modified Dulbecco's Medium, and mixtures thereof. In one embodiment, the basal salt nutrient solution is an approximately 50:50 mixture of DMEM and Ham's F12.

10 **[0029]** As used herein, the term "member of the TGF- $\beta$  family" refers to growth factors that are generally characterized by one of skill in the art as belonging to the TGF- $\beta$  family, either due to homology with known members of the TGF- $\beta$  family, or due to similarity in function with known members of the TGF- $\beta$  family. In certain 20 embodiments, the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4. In one embodiment, the member of the TGF- $\beta$  family is Activin A.

25 **[0030]** As used herein, the term "activator of an FGF receptor" refers to growth factors that are generally characterized by one of skill in the art as belonging to the FGF family, either due to homology with known members of the FGF family, or due to similarity in function with known members of the FGF family. In certain 30 embodiments, the activator of an FGF receptor is an FGF, such as, but not limited to  $\alpha$ -FGF and FGF2.

**[0031]** As used herein, the term "activator of IGF-1R" refers to mitogens that play a pivotal role in regulating cell proliferation, differentiation, and apoptosis. The effects of an activator of IGF-1R are typically mediated through IGF-1R, although they

can be mediated through other receptors. The IGF-1R is also involved in cell transformation induced by tumor virus proteins and oncogene products, and the interaction is regulated by a group of specific binding proteins (IGFBPs). In addition, a large group of IGFBP proteases hydrolyze IGFBPs, resulting in the release of bound

5 IGFs that then resume their ability to interact with IGF-1R. For the purpose of this invention, the ligands, the receptors, the binding proteins, and the proteases are all considered to be activators of IGF-1R. In one embodiment, the activator of IGF-1R is insulin. In one embodiment, the activator of IGF-1R is IGF-1, or IGF-2. In a further embodiment, the IGF-1 is an IGF-1 analog. Non-limiting examples of IGF-1 analogs

10 include LongR<sup>3</sup>IGF-1, Des(1-3)IGF-1, [Arg<sup>3</sup>]IGF-1, [Ala<sup>31</sup>]IGF-1, Des(2,3)[Ala<sup>31</sup>]IGF-1, [Leu<sup>24</sup>]IGF1, Des(2,3)[Leu<sup>24</sup>]IGF-1, [Leu<sup>60</sup>]IGF-1, [Ala<sup>31</sup>][Leu<sup>60</sup>]IGF-1, [Leu<sup>24</sup>][Ala<sup>31</sup>]IGF-1, and combinations thereof. In a further embodiment, the IGF-1 analog is LongR<sup>3</sup>IGF-1, a recombinant analog of human insulin growth factor-1 (JRH Biosciences; Yandell *et al.*, 2004, BioProcess Intl., 56-64). LongR<sup>3</sup>IGF-1 has greatly

15 decreased affinity for IGFBPs, and may therefore be more bioactive in cell culture.

**[0032]** It is also noted that insulin, IGF-1, and IGF-2 are activators of the PI3K pathway. Accordingly, it is contemplated herein that other regulators of the PI3K pathway may act in the same or similar fashion; either in conjunction with or independently of insulin and IGFs. Therefore, the invention provides in certain

20 embodiments that the compositions consist essentially of a basal salt nutrient solution, an activator of the PI3K pathway, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free. In certain embodiments, the activator of the PI3K pathway is an activator of IGF-1R, and the composition further comprises a second activator of the

25 PI3K pathway that may act through a separate receptor.

**[0033]** As used herein, the term “insulin” refers to a protein that binds to the insulin receptor and can induce signaling through the receptor. Alternatively, insulin can signal through other receptors, such as, but not limited to, IGF-1R. The term “insulin” encompasses a protein having the polypeptide sequence of native human insulin, or of other mammalian insulin, or of any homologs to these sequences, and includes any zinc containing compound that may be used in place of insulin to give substantially the same results as insulin. Additionally, the term insulin encompasses polypeptide fragments that are capable of binding to the insulin or IGF-1 receptor and inducing signaling through the receptor(s).

[0034] The invention further encompasses variants of naturally occurring members of the TGF- $\beta$  family, and of naturally occurring activators of an FGF receptor and of IGF-1R. As used herein, the term "variant" includes chimeric or fusion polypeptides, homologs, analogs, orthologs, and paralogs.

5 [0035] The invention also provides chimeric or fusion polypeptides. As used herein, a "chimeric polypeptide" or "fusion polypeptide" comprises at least a portion of a member of the reference polypeptide operatively linked to a second, different polypeptide. The second polypeptide has an amino acid sequence corresponding to a polypeptide which is not substantially identical to the reference polypeptide, and which

10 is derived from the same or a different organism. With respect to the fusion polypeptide, the term "operatively linked" is intended to indicate that the reference polypeptide and the second polypeptide are fused to each other so that both sequences fulfill the proposed function attributed to the sequence used. The second polypeptide can be fused to the N-terminus or C-terminus of the reference polypeptide. For

15 example, in one embodiment, the fusion polypeptide is a GST-IGF-1 fusion polypeptide in which an IGF-1 sequence is fused to the C-terminus of the GST sequences. Such fusion polypeptides can facilitate the purification of recombinant polypeptides. In another embodiment, the fusion polypeptide can contain a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian

20 host cells), expression and/or secretion of a polypeptide can be increased through use of a heterologous signal sequence.

[0036] In addition to fragments and fusion polypeptides, the present invention includes homologs and analogs of naturally occurring polypeptides. "Homologs" are defined herein as two nucleic acids or polypeptides that have similar, or "identical,"

25 nucleotide or amino acid sequences, respectively. Homologs include allelic variants, orthologs, paralogs, agonists, and antagonists as defined hereafter. The term "homolog" further encompasses nucleic acid molecules that differ from a reference nucleotide sequence due to degeneracy of the genetic code and thus encode the same polypeptide as that encoded by the reference nucleotide sequence. As used herein, "naturally occurring" refers to a nucleic or amino acid sequence that occurs in nature.

30 [0037] An agonist of a polypeptide can retain substantially the same, or a subset, of the biological activities of the polypeptide. An antagonist of a polypeptide can inhibit one or more of the activities of the naturally occurring form of the polypeptide.

[0038] In one embodiment, the pluripotent cells are contacted with an effective amount of an activator of an FGF receptor, an effective amount of an activator of IGF-1R, and optionally with an effective amount of a member of the TGF- $\beta$  family. As used herein, the term "effective amount" of a compound refers to that concentration of the compound that is sufficient in the presence of the remaining components of the defined medium to effect the stabilization of the pluripotent cell in culture for greater than one month in the absence of a feeder cell and in the absence of serum or serum replacement. This concentration is readily determined by one of ordinary skill in the art.

[0039] It is contemplated that the defined medium comprises a basal salt nutrient solution, and further comprises an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family. In addition, other minor components such as L-glutamine, non-essential amino acids, amino acids, lipids, ascorbic acid, trace elements, antibiotics,  $\beta$ -Mercaptoethanol, and similar components may be present.

[0040] It is understood that at different points during culturing the pluripotent cells, various components may be added to the cell culture such that the medium can contain components other than those described herein. However, it is contemplated that at least at one point during the preparation of the culture, or during the culture of the pluripotent cells, the defined medium can comprise a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family.

[0041] In particular embodiments of the invention, the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4. In a further embodiment, the member of the TGF- $\beta$  family is Activin A. It is contemplated that Nodal is initially present at a concentration of approximately 0.1 ng/ml to approximately 100 ng/ml, more preferably approximately 0.5 ng/ml to approximately 75 ng/ml, more preferably approximately 1 ng/ml to approximately 50 ng/ml, or more preferably approximately 2.5 ng/ml to approximately 5 ng/ml.

[0042] It is contemplated that Activin A is initially present at a concentration of approximately 0.01 ng/ml to approximately 1000 ng/ml, more preferably approximately 0.1 ng/ml to approximately 100 ng/ml, more preferably approximately

0.1 ng/ml to approximately 10 ng/ml, or most preferably at a concentration of approximately 1 ng/ml.

[0043] It is contemplated that TGF- $\beta$  is initially present at a concentration of approximately 0.01 ng/ml to approximately 100 ng/ml, more preferably approximately 5 0.1 ng/ml to approximately 50 ng/ml, or more preferably approximately 0.1 ng/ml to approximately 20 ng/ml.

[0044] It is contemplated that BMP4 is initially present at a concentration of approximately 0.01 ng/ml to approximately 100 ng/ml, more preferably approximately 10 0.05 ng/ml to approximately 10 ng/ml, or more preferably approximately 0.05 ng/ml to approximately 0.01 ng/ml.

[0045] It is contemplated that the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor. In one embodiment, the activator of IGF-1R is insulin. It is contemplated that insulin is initially present at a concentration of approximately 1  $\mu$ g/ml to approximately 1000  $\mu$ g/ml, more preferably 15 approximately 5  $\mu$ g/ml to approximately 100  $\mu$ g/ml, more preferably approximately 10  $\mu$ g/ml to approximately 50  $\mu$ g/ml, or most preferably at a concentration of approximately 20  $\mu$ g/ml. In another embodiment, the insulin like growth factor is IGF-1 or IGF-2. In one embodiment, the insulin-like growth factor is IGF-1, which can be LongR<sup>3</sup>IGF-1. It is contemplated that LongR<sup>3</sup>IGF-1 is initially present at a 20 concentration of approximately 1 ng/ml to approximately 1000 ng/ml, more preferably approximately 5 ng/ml to approximately 500 ng/ml, more preferably approximately 50 ng/ml to approximately 500 ng/ml, more preferably approximately 100 ng/ml to approximately 300 ng/ml, or at a concentration of approximately 100 ng/ml.

[0046] In one embodiment, the activator of an FGF receptor is FGF2. It is 25 contemplated that FGF2 is initially present at a concentration of approximately 0.1 ng/ml to approximately 100 ng/ml, more preferably approximately 0.5 ng/ml to approximately 50 ng/ml, more preferably approximately 1 ng/ml to approximately 25 ng/ml, more preferably approximately 1 ng/ml to approximately 12 ng/ml, or most preferably at a concentration of approximately 8 ng/ml.

[0047] In particular embodiments of the invention, the serum albumin is 30 selected from bovine serum albumin and human serum albumin. It is contemplated that the serum albumin is initially present at a concentration of from approximately 0.02-5.0%, from approximately 0.05-2% or from approximately 0.1-0.5%.

[0048] It is contemplated that the composition can further comprise trace elements. Trace elements can be purchased commercially, for example, from Mediatech. Non-limiting examples of trace elements include AlCl<sub>3</sub>, AgNO<sub>3</sub>, Ba(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>, CdCl<sub>2</sub>, CdSO<sub>4</sub>, CoCl<sub>2</sub>, CrCl<sub>3</sub>, Cr<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, CuSO<sub>4</sub>, ferric citrate, GeO<sub>2</sub>, KI, 5 KBr, Li, molybdic acid, MnSO<sub>4</sub>, MnCl<sub>2</sub>, NaF, Na<sub>2</sub>SiO<sub>3</sub>, NaVO<sub>3</sub>, NH<sub>4</sub>VO<sub>3</sub>, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, NiSO<sub>4</sub>, RbCl, selenium, Na<sub>2</sub>SeO<sub>3</sub>, H<sub>2</sub>SeO<sub>3</sub>, selenite·2Na, selenomethionone, SnCl<sub>2</sub>, ZnSO<sub>4</sub>, ZrOCl<sub>2</sub>, and mixtures and salts thereof. If selenium, selenite or selenomethionone is present, it is at a concentration of approximately 0.002 to approximately 0.02 mg/L.

10 [0049] It is contemplated that amino acids can be added to the defined media. Non-limiting examples of such amino acids are Glycine, L-Alanine, L-Alanyl-L-Glutamine, L-Glutamine/Glutamax, L-Arginine hydrochloride, L-Asparagine-H<sub>2</sub>O, L-Aspartic acid, L-Cysteine hydrochloride-H<sub>2</sub>O, L-Cystine 2HCl, L-Glutamic Acid, L-Histidine hydrochloride-H<sub>2</sub>O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L- 15 Methionine, L-Phenylalanine, L-Proline, L-Hydroxyproline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, and L-Valine. In certain embodiments, the amino acid is L-Isoleucine, L-Phenylalanine, L-Proline, L-Hydroxyproline, L-Valine, and mixtures thereof.

[0050] It is also contemplated that the defined medium can comprise ascorbic acid. Preferably ascorbic acid is present at an initial concentration of approximately 1 mg/L to approximately 1000 mg/L, or from approximately 2 mg/L to approximately 500 mg/L, or from approximately 5 mg/L to approximately 100 mg/L, or from approximately 10 mg/L to approximately 100 mg/L or approximately at 50 mg/L.

20 [0051] In certain embodiments, the composition comprises an inactivator of Notch signaling. As used herein, an “inactivator of Notch signaling” refers to an agent that antagonizes the activity of one or more Notch proteins or any of their upstream or downstream signaling components through any of its possible signaling pathways. The compound used to inactivate Notch signaling can be any compound known in the art, or later discovered. Non-limiting examples of inactivators of Notch signaling include 25 dominant-negative, truncated Delta or Serrate molecules that lack intracellular domains, and inhibitors of the gamma-secretase complex.

30 [0052] In certain embodiments, the defined medium can comprise a growth hormone. It is currently contemplated that in certain embodiments, the growth hormone present in the defined medium will be of the same species as the pluripotent

mammalian cell that is cultured with the defined media. Thus, for example, if a human cell is cultured, the growth hormone is human growth hormone. The use of growth hormone that is from a species different than the cultured cells is also contemplated. Preferably growth hormone is present at an initial concentration of 5 approximately 0.001 ng/ml to approximately 1000 ng/ml, more preferably approximately 0.001 ng/ml to approximately 250 ng/ml, or more preferably approximately 0.01 ng/ml to approximately 150 ng/ml.

[0053] It is preferred that the defined media of the invention is essentially free of serum and serum replacement, and is essentially serum free. As used herein, 10 "essentially serum free" refers to a medium that does not contain serum or serum replacement, or that contains essentially no serum or serum replacement. As used herein, "essentially" means that a de minimus or reduced amount of a component, such as serum or serum replacement, may be present that does not eliminate the improved bioactive culturing capacity of the medium or environment. For example, essentially 15 serum free medium or environment can contain less than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1% serum wherein the presently improved bioactive maintenance capacity of the medium or environment is still observed. In preferred embodiments of the present invention, the essentially serum free medium does not contain serum or serum replacement, or only contains trace amounts of serum or serum replacement from the 20 isolation of components of the serum or serum replacement that are added to the defined media.

[0054] In one embodiment, the pluripotent cells are contacted with the defined media of the invention in the absence of serum or serum replacement, and in the absence of a feeder cell layer, such that the cells are maintained in an undifferentiated 25 state for at least one month. Pluripotency can be determined through characterization of the cells with respect to surface markers, transcriptional markers, karyotype, and ability to differentiate to cells of the three germ layers. These characteristics are well known to those of ordinary skill in the art.

[0055] As used herein when referring to a cell, cell line, cell culture, or 30 population of cells, the term "isolated" refers to being substantially separated from the natural source of the cells such that the cell, cell line, cell culture, or population of cells are capable of being cultured *in vitro*. In addition, the term "isolating" is used to refer to the physical selection of one or more cells out of a group of two or more cells,

wherein the cells are selected based on cell morphology and/or the expression of various markers.

[0056] As used herein, the term “express” refers to the transcription of a polynucleotide or translation of a polypeptide in a cell, such that levels of the molecule are measurably higher in a cell that expresses the molecule than they are in a cell that does not express the molecule. Methods to measure the expression of a molecule are well known to those of ordinary skill in the art, and include without limitation, Northern blotting, RT-PCR, *in situ* hybridization, Western blotting, and immunostaining.

[0057] As used herein, the term “contacting” (*i.e.*, contacting a cell *e.g.* a pluripotent cell, with a compound) is intended to include incubating the compound and the cell together *in vitro* (*e.g.*, adding the compound to cells in culture). The term “contacting” is not intended to include the *in vivo* exposure of cells to a defined cell medium comprising an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, that may occur naturally in a subject (*i.e.*, exposure that may occur as a result of a natural physiological process). The step of contacting the cell with a defined cell medium comprising an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, can be conducted in any suitable manner. For example, the cells may be treated in adherent culture, or in suspension culture. It is understood that the cells contacted with the defined medium can be further treated with a cell differentiation environments to stabilize the cells, or to differentiate the cells.

[0058] The compositions and methods described herein have several useful features. For example, the compositions and methods described herein are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as neurodegenerative disorders, diabetes mellitus or renal failure, such as by the development of pure tissue or cell types.

[0059] As used herein, the term “differentiate” refers to the production of a cell type that is more differentiated than the cell type from which it is derived. The term therefore encompasses cell types that are partially and terminally differentiated.

**[0060]** In certain embodiments of the present invention, the term “enriched” refers to a cell culture that contains more than approximately 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the desired cell lineage.

**[0061]** The cell types that differentiate from embryonic stem cells have several 5 uses in various fields of research and development including but not limited to drug discovery, drug development and testing, toxicology, production of cells for therapeutic purposes as well as basic science research. These cell types express molecules that are of interest in a wide range of research fields. These include the molecules known to be required for the functioning of the various cell types as 10 described in standard reference texts. These molecules include, but are not limited to, cytokines, growth factors, cytokine receptors, extracellular matrix, transcription factors, secreted polypeptides and other molecules, and growth factor receptors.

**[0062]** In one embodiment, the pluripotent cell is a human cell. As used herein, the term “pluripotent human cell” encompasses pluripotent cells obtained from 15 human embryos, fetuses, or adult tissues. In one preferred embodiment, the pluripotent human cell is a human pluripotent embryonic stem cell. In another embodiment the pluripotent human cell is a human pluripotent fetal stem cell, such as a primordial germ cell. In another embodiment the pluripotent human cell is a human pluripotent adult stem cell. As used herein, the term “pluripotent” refers to a cell 20 capable of at least developing into one of ectodermal, endodermal, and mesodermal cells. As used herein the term “pluripotent” refers to cells that are totipotent and multipotent. As used herein, the term “totipotent cell” refers to a cell capable of developing into all lineages of cells. The term “multipotent” refers to a cell that is not terminally differentiated. As also used herein, the term “multipotent” refers to a cell 25 that, without manipulation (*i.e.*, nuclear transfer or dedifferentiation inducement), is incapable of forming differentiated cell types derived from all three germ layers (mesoderm, ectoderm, and endoderm), or in other words, is a cell that is partially differentiated. The pluripotent human cell can be selected from the group consisting of a human embryonic stem (ES) cell; a human inner cell mass (ICM)/epiblast cell; a 30 human primitive ectoderm cell, such as an early primitive ectoderm cell (EPL); a human primordial germ (EG) cell; and a human teratocarcinoma (EC) cell. The human pluripotent cells of the present invention can be derived using any method known to those of skill in the art. For example, the human pluripotent cells can be produced using de-differentiation and nuclear transfer methods. Additionally, the human

ICM/epiblast cell or the primitive ectoderm cell used in the present invention can be derived *in vivo* or *in vitro*. EPL cells may be generated in adherent culture or as cell aggregates in suspension culture, as described in PCT Publication No. WO 99/53021. Furthermore, the human pluripotent cells can be passaged using any method known to 5 those of skill in the art, including, manual passaging methods, and bulk passaging methods such as antibody selection and protease passaging.

**[0063]** In certain embodiments, the embryonic stem cell of the invention has a normal karyotype, while in other embodiments, the embryonic stem cell has an abnormal karyotype. In one embodiment, a majority of the embryonic stem cells have 10 an abnormal karyotype. It is contemplated that greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or greater than 95% of metaphases examined will display an abnormal karyotype. In certain embodiments, the abnormal karyotype is evident after the cells have been cultured for greater than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20 passages. In one embodiment, the abnormal karyotype comprises a trisomy of at 15 least one autosomal chromosome, wherein the autosomal chromosome is selected from the group consisting of chromosomes 1, 7, 8, 12, 14, and 17. In another embodiment, the abnormal karyotype comprises a trisomy of more than one autosomal chromosome, wherein at least one of the more than one autosomal chromosomes is selected from the group consisting of chromosomes 1, 7, 8, 12, 14, and 17. In one embodiment, the 20 autosomal chromosome is chromosome 12 or 17. In another embodiment, the abnormal karyotype comprises an additional sex chromosome. In one embodiment, the karyotype comprises two X chromosomes and one Y chromosome. It is also contemplated that translocations of chromosomes may occur, and such translocations are encompassed within the term “abnormal karyotype.” Combinations of the 25 foregoing chromosomal abnormalities and other chromosomal abnormalities are also encompassed by the invention.

**[0064]** It is contemplated that the pluripotent cells of the invention can be differentiated through contact with a cell differentiation environment. As used herein, the term “cell differentiation environment” refers to a cell culture condition wherein 30 the pluripotent cells are induced to differentiate, or are induced to become a human cell culture enriched in differentiated cells. Preferably, the differentiated cell lineage induced by the growth factor will be homogeneous in nature. The term “homogeneous,” refers to a population that contains more than approximately 50%,

60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the desired cell lineage.

**[0065]** A cell differentiating medium or environment may be utilized to partially, terminally, or reversibly differentiate the pluripotent cells of the present invention. In accordance with the invention the medium of the cell differentiation environment may contain a variety of components including, for example, KODMEM medium (Knockout Dulbecco's Modified Eagle's Medium), DMEM, Ham's F12 medium, FBS (fetal bovine serum), FGF2 (fibroblast growth factor 2), KSR, or hLIF (human leukemia inhibitory factor). The cell differentiation environment can also contain supplements such as L-Glutamine, NEAA (non-essential amino acids), P/S (penicillin/streptomycin), N2, and  $\beta$ -mercaptoethanol ( $\beta$ -ME). It is contemplated that additional factors may be added to the cell differentiation environment, including, but not limited to, fibronectin, laminin, heparin, heparin sulfate, retinoic acid, members of the epidermal growth factor family (EGFs), members of the fibroblast growth factor family (FGFs) including FGF2 and/or FGF8, members of the platelet derived growth factor family (PDGFs), transforming growth factor (TGF)/ bone morphogenetic protein (BMP)/ growth and differentiation factor (GDF) factor family antagonists including but not limited to noggin, follistatin, chordin, gremlin, cerberus/DAN family proteins, ventropin, high dose activin, and amnionless. TGF/BMP/GDF antagonists could also be added in the form of TGF/BMP/GDF receptor-Fc chimeras. Other factors that may be added include molecules that can activate or inactivate signaling through Notch receptor family, including but not limited to proteins of the Delta-like and Jagged families as well as inhibitors of Notch processing or cleavage. Other growth factors may include members of the insulin like growth factor family (IGF), insulin, the wingless related (WNT) factor family, and the hedgehog factor family. Additional factors may be added to promote mesendoderm stem/progenitor, endoderm stem/progenitor, mesoderm stem/progenitor, or definitive endoderm stem/progenitor proliferation and survival as well as survival and differentiation of derivatives of these progenitors.

**[0066]** In certain embodiments, the cell culture environment comprises plating the cells in an adherent culture. As used herein, the terms "plated" and "plating" refer to any process that allows a cell to be grown in adherent culture. As used herein, the term "adherent culture" refers to a cell culture system whereby cells are cultured on a solid surface, which may in turn be coated with a solid substrate that may in turn be

coated with another surface coat of a substrate, such as those listed below, or any other chemical or biological material that allows the cells to proliferate or be stabilized in culture. The cells may or may not tightly adhere to the solid surface or to the substrate. The substrate for the adherent culture may comprise any one or combination 5 of polyornithine, laminin, poly-lysine, purified collagen, gelatin, fibronectin, tenascin, vitronectin, entactin, heparin sulfate proteoglycans, poly glycolytic acid (PGA), poly lactic acid (PLA), and poly lactic-glycolic acid (PLGA). Furthermore, the substrate for the adherent culture may comprise the matrix laid down by a feeder layer, or laid down 10 by the pluripotent human cell or cell culture. As used herein, the term "extracellular matrix" encompasses solid substrates such as but not limited to those described above, as well as the matrix laid down by a feeder cell layer or by the pluripotent human cell or cell culture. In one embodiment, the cells are plated on matrigel coated plates. In another embodiment, the cells are plated on fibronectin coated plates. In certain 15 embodiments, if the cells are plated on fibronectin, the plates are prepared by coating with 10 µg/ml human plasma fibronectin (Invitrogen, #33016-015), diluted in tissue grade water, for 2-3 hours at room temperature.

**[0067]** The methods of the present invention contemplate that cells are cultured in conditions that are essentially free of a feeder cell or feeder layer. As used herein, a "feeder cell" is a cell that grows *in vitro*, that is co-cultured with a target cell and 20 stabilizes the target cell in its current state of differentiation. As used herein, a "feeder cell layer" can be used interchangeably with the term "feeder cell." As used herein, the term "essentially free of a feeder cell" refers to tissue culture conditions that do not contain feeder cells, or that contain a *de minimus* number of feeder cells. By "*de minimus*", it is meant that number of feeder cells that are carried over to the instant 25 culture conditions from previous culture conditions where the pluripotent cells may have been cultured on feeder cells. In one embodiment of the above method, conditioned medium is obtained from a feeder cell that stabilizes the target cell in its current state of differentiation. In another embodiment, the defined medium is a non-conditioned medium, which is a medium that is not obtained from a feeder cell.

**[0068]** As used herein, the term "stabilize" refers to the differentiation state of 30 a cell. When a cell or cell population is stabilized, it will continue to proliferate over multiple passages in culture, and preferably indefinitely in culture; additionally, each cell in the culture is preferably of the same differentiation state, and when the cells divide, typically yield cells of the same cell type or yield cells of the same

differentiation state. Preferably, a stabilized cell or cell population does not further differentiate or de-differentiate if the cell culture conditions are not altered, and the cells continue to be passaged and are not overgrown. Preferably the cell that is stabilized is capable of proliferation in the stable state indefinitely, or for at least more than 2 passages. Preferably, it is stable for more than 5 passages, more than 10 passages, more than 15 passages, more than 20 passages, more than 25 passages, or most preferably, it is stable for more than 30 passages. In one embodiment, the cell is stable for greater than approximately 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, or 11 months of continuous passaging. In another embodiment, the cell is stable for greater than approximately 1 year of continuous passaging. In one embodiment, stem cells are maintained in culture in a pluripotent state by routine passage in the defined medium until it is desired that they be differentiated. As used herein, the term "proliferate" refers to an increase in the number cells in a cell culture.

15 [0069] The progression of the hESC culture to the desired cell lineage, or its maintenance in an undifferentiated state can be monitored by quantitating expression of marker genes characteristic of the desired cell lineage as well as the lack of expression of marker genes characteristic of hESCs and other cell types. One method of quantitating gene expression of such marker genes is through the use of quantitative 20 PCR (Q-PCR). Methods of performing Q-PCR are well known in the art. Other methods that are known in the art can also be used to quantitate marker gene expression. Marker gene expression can be detected by using antibodies specific for the marker gene of interest.

25 [0070] Using the methods described herein, compositions comprising the desired cell lineage that are substantially free of other cell types can be produced. Alternatively, compositions comprising mixtures of hESCs and the desired cell lineage can be produced.

30 [0071] In some embodiments of the present invention, cells of the desired cell lineage can be isolated by using an affinity tag that is specific for such cells. One example of an affinity tag specific for a target cell is an antibody that is specific to a marker polypeptide that is present on the cell surface of the target cell but which is not substantially present on other cell types that would be found in a cell culture produced by the methods described herein.

[0072] It is contemplated that the pluripotent cells can be passaged using enzymatic, non-enzymatic, or manually dissociation methods prior to and/or after contact with the defined medium of the invention. Non-limiting examples of enzymatic dissociation methods include the use of proteases such as trypsin, 5 collagenase, dispase, and accutase. In one embodiment, accutase is used to passage the contacted cells. When enzymatic passaging methods are used, the resultant culture can comprise a mixture of singlets, doublets, triplets, and clumps of cells that vary in size depending on the enzyme used. A non-limiting example of a non-enzymatic dissociation method is a cell dispersal buffer. Manual passaging techniques have been 10 well described in the art, such as in Schulz *et al.*, 2004, *Stem Cells*, 22(7):1218-38. The choice of passaging method is influenced by the choice of extracellular matrix, and is easily determined by one of ordinary skill in the art.

[0073] Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those 15 publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

## EXAMPLES

20 Methods

[0074] The human embryonic stem cell lines BG03 and BG01v (BresaGen, Inc., Athens, GA) were used in this work. The BG01v hESC line is a karyotypically variant cell line, which exhibits stable karyotype containing specific trisomies (karyotype: 49, XXY,+12,+17). Parent cultures were maintained as described 25 previously (Schulz *et al.*, 2003, *BMC Neurosci.*, 4:27; Schulz *et al.*, 2004, *Stem Cells*, 22(7):1218-38; Rosler *et al.*, 2004, *Dev. Dynamics*, 229:259-274; Brimble *et al.*, 2004, *Stem Cells Dev.*, 13:585-596). Briefly, the cells were grown in dishes coated with Matrigel or fibronectin, in conditioned media from MEFs (MEF-CM) comprising DMEM:F12 with 20% KSR, 8 ng/ml FGF2, 2 mM L-Glutamine, 1x non-essential 30 amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol (Sigma) with collagenase passaging.

[0075] The defined culture media tested herein comprised DMEM/F12, 2 mM L-glutamine, 1x non-essential amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 10  $\mu$ g/ml transferrin (all from Invitrogen) 0.1 mM  $\beta$ -mercaptoethanol (Sigma), and the

experimental variables. The variables included the growth factors recombinant human insulin (Invitrogen), recombinant LongR<sup>3</sup>-IGF1 (JRH Biosciences), recombinant FGF2 (Sigma), Activin A (R&D Systems), recombinant TGF $\beta$  (R&D systems), recombinant BMP4 (R&D systems), and recombinant Nodal (R&D systems). LongR<sup>3</sup>-IGF1 is a 5 modified version of IGF1 that has reduced affinity for IGF1 binding proteins, some of which are expressed in hESCs.

10 [0076] Additionally, the media further comprises trace elements such as selenium, ascorbic acid, amino acids, and a source of lipids.

15 [0077] Matrigel coated dishes were prepared by diluting Growth Factor Reduced BD matrigel matrix (BD Biosciences) to a final concentration of 1:30 in cold DMEM/F-12. 1 ml/35 mm dish was used to coat dishes for 1-2 hours at room temperature or at least overnight at 4°C. Plates were stored up to one week at 4°C. Matrigel solution was removed immediately before use.

20 [0078] For the tested conditions, parent cultures were plated into 6-well dishes for comparison of multiple conditions. Cultures were either plated directly into the test conditions, or were plated with MEF-CM for one day to promote adherence, prior to changing the media to the test conditions. The cultures were assessed every day and graded based on morphological criteria 4 to 5 days after plating. The grading scale of 1 to 5 involved examining the whole culture and assessing overall proportion of undifferentiated colonies, their relative size, and proportion of colonies or parts of colonies exhibiting obvious differentiation. Grade 5 indicates "ideal" cultures, with large undifferentiated colonies and negligible differentiation. Grade 4 indicates a very good culture, but with some obvious differentiation. Grade 3 indicates acceptable cultures, but with around half the colonies exhibiting obvious differentiation. Grade 2 cultures are predominantly differentiated, with occasional putative undifferentiated cells. Grade 1 cultures contain differentiated colonies or the cultures did not adhere or did not survive. Cultures that exhibited good expansion of undifferentiated cells were 25 passaged to assess the generation of stable sublines in these semi-defined media.

30

### Example 1

#### *Expansion of hESCs in FGF2/insulin or FGF2/<sup>LR</sup>-IGF1 conditioned medium*

35 [0079] BG01v cells were cultured as described above. Specifically, BG01v cells were plated to 6-well dishes that had been coated with matrigel. Cells were plated at a concentration such that well-defined and isolated colonies are visible after

4-5 days. The cells were cultured for one day in 20% KSR MEF-CM to ensure effective plating. After one day, the media was changed to the experimental conditions shown in Table 1. The base media (1:1 DMEM/F12, 2 mM L-glutamine, 1x non-essential amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol, 4 ng/ml FGF2) had been conditioned on MEFs for 24 hours. The components that were added to this media to generate the experimental conditions were an additional 4 ng/ml FGF2 (8ng/ml final), and 20  $\mu$ g/ml insulin, 50 ng/ml LongR<sup>3</sup>-IGF1, 100 ng/ml Activin A, and/or 0.2% BSA in the indicated combinations.

[0080] Grading of the cultures on day 4 indicated that conditions containing 8 ng/ml FGF2 and 0.2 % BSA in combination with either 20  $\mu$ g/ml insulin or 50 ng/ml LongR<sup>3</sup>-IGF1 supported the expansion of undifferentiated BG01v cells (Table 1, Fig. 1A-D).

Table 1

Conditions	Insulin	FGF2	LR <sup>3</sup> -IGF1	Activin	BSA	Colony count	Colony grade
	20 $\mu$ g/ml	8 ng/ml	50 ng/ml	100 ng/ml	0.20%		
1			+			7	1
2			+			12	3
3	+	+				13	2
4	+	+				45	4 to 5
5	+	+		+		3	1
6	+	+		+	+	7	2
7	+		+			15	2
8	+	+				50	4 to 5
9	+	+		+		3	1
10	+	+		+	+	16	1 to 2

[0081] In the presence of insulin and LongR<sup>3</sup>-IGF1, there were 45 and 50 colonies, respectively, with grades 4 to 5. In the same conditions, in the absence of BSA, the grade of the culture was only 2, with far fewer colonies surviving. This indicated the importance of a serum albumin component. The addition of 100 ng/ml Activin A appeared to induce differentiation as well as cell death, with only a few colonies surviving at this concentration, and very few putative undifferentiated cells remaining. A few undifferentiated colonies were present in conditions containing 8 ng/ml FGF (without insulin or LongR<sup>3</sup>-IGF1), although these were smaller than when

insulin or LongR<sup>3</sup>-IGF1 was included. The morphologies of colonies grown in some of the different media conditions are shown in Figures 1A-D.

### Example 2

5 *Expansion of hESCs in FGF2/insulin or FGF2/ LongR<sup>3</sup>-IGF1 medium*

[0082] BG01v cells were cultured as described above. Specifically, BG01v cells were plated to 6-well dishes that had been coated with matrigel. The cells were plated directly in the experimental conditions indicated in Table 2. The base media for conditions 1 and 2 (1:1 DMEM/F12, 2 mM L-glutamine, 1x non-essential amino acids, 10 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol, 4 ng/ml FGF2, 10  $\mu$ g/ml transferrin) had been conditioned on MEFs for 24 hours, and an additional 4 ng/ml FGF2 (8 ng/ml final concentration) and 0.2% BSA were added prior to use. Condition 1 included 20  $\mu$ g/ml insulin and condition 2 included 50 ng/ml LongR<sup>3</sup>-IGF1. For conditions 3-12, the base media was not conditioned. The 15 components that were added to the base media were an additional 4 ng/ml FGF2 (8 ng/ml final), and 20  $\mu$ g/ml insulin, 50 ng/ml LongR<sup>3</sup>-IGF1, 100 ng/ml Activin A, and/or 0.2% BSA in the indicated combinations.

Table 2

Conditions

	Insulin	FGF2	LR <sup>3</sup> -IGF1	Activin	BSA	Colony count	Colony grade
	20 $\mu$ g/ml	8 ng/ml	50 ng/ml	100 ng/ml	0.2%		

CM

1	+	+			+	~100	4 to 5
2		+	+		+	~100	4 to 5

Non-CM

3	+	+			+	~250	4 to 5
4	+				+	~250	3
5		+			+	~200	4
6	+			+	+	~50	1 to 2
7	+	+		+	+	~100	2 to 3
8	+		+		+	~200 to 250	4 to 5
9			+		+	~200	2 to 3
10			+	+	+	~100	1 to 3
11	+	+		+	+	~100	1 to 3
12	+			+	+	~100	0 to 1

[0083] The cultures were graded at day 4. Wells 1 and 2, where the base media had been conditioned on MEFs, exhibited large undifferentiated colonies of hESCs. However, when cells were grown in these conditions without conditioning of the base media (wells 3 and 8, respectively), more than double the number of colonies 5 were observed, whilst the grade of the culture was the same. This indicated there were factors in this conditioned base media that negatively affected the plating of hESCs. Conditions containing 8 ng/ml FGF2 and 20 µg/ml insulin, or 50 ng/ml LongR<sup>3</sup>-IGF1, enabled effective proliferation of undifferentiated colonies and were graded at 4 to 5. Omitting FGF2 from these conditions (Wells 4 and 9, respectively), led to a lower 10 grading (3 and 2 to 3, respectively), with the cultures appearing to contain much higher levels of differentiation. Similarly, conditions with 8 ng/ml FGF2, (without insulin, or LongR<sup>3</sup>-IGF1 (Well 5)) were given a grading of 4, and while primarily undifferentiated, had not proliferated as extensively. Therefore it appeared that a combination of either 20 µg/ml insulin or 50 ng/ml LongR<sup>3</sup>-IGF1 with 8 ng/ml FGF2 15 was required to generate the best cultures. The presence of 100 ng/ml Activin A appeared to induce differentiation in all conditions, with these cultures being graded from 1 to 3. The morphologies of colonies grown in some of the different media conditions are shown in Figures 2A-G.

20 **Example 3**

*Titration of components and growth factors*

[0084] Several experiments were performed to titrate components of this defined media, in order to identify the optimal concentrations of individual components. Furthermore, in Examples 1 and 2, it appeared that neural precursors 25 were the predominant cell type that was generated when hESCs spontaneously differentiated. These were observed as rosettes and clusters of rosettes, typically in the center of otherwise undifferentiated hESC colonies. This was consistent with the apparently default differentiation of hESCs to neural lineages in the absence of serum or serum replacement (Schulz *et al*, 2004, Stem Cells, 22:1218-1238; Vallier *et al.*, 30 2004, Dev Biol., 275(2):403-21). The effects of members of the TGF $\beta$  family on inhibition of neuronal differentiation were tested in these cultures. The factors tested included Activin A, BMP4, TGF $\beta$ , and Nodal.

Table 3

	FGF2 8 ng/ml	LR <sup>3</sup> -IGF1 100 ng/ml	Activin 1 ng/ml	BMP4 ng/ml	TGF $\beta$ ng/ml	Nodal ng/ml	BSA 0.2 %	Grade	p1 grade
A	+	50	0.1				+	3 to 4	
	+	50	0.5				+	3 to 4	
	+	50	+				+	3 to 4	
	+	50	5				+	4	
	+	50	10				+	3 to 4	
B	+	50					+	2 to 3	
	+	50		0.05			+	3 to 4	2
	+	50		0.1			+	3 to 4	2
	+	50		1			+	3	
	+	50		5			+	1	
	+	50		10			+	1	
C	+	50	+				+	4	
	+	+	+				+	4	3 to 4
	+	150	+				+	4	
	+	200	+				+	4	3 to 4
	+	300	+				+	4	3 to 4
	+	500	+				+	4	3
D	+	+		0.1			+	3 to 4	2
	+	+		1			+	3 to 4	
	+	+		2.5			+	3 to 4	2
	+	+		5			+	3 to 4	
	+	+		10			+	3 to 4	2
	+	+		20			+	3 to 4	1
E	+	+			1	+	+	3 to 4	2
	+	+			2.5	+	+	4	2
	+	+			5	+	+	4	2 to 3
	+	+			10	+	+	3	3
	+	+			20	+	+	3	
	+	+			50	+	+	3	
F	+	+	+			0.02		1	
	+	+	+			0.05		1	
	+	+	+			0.1		3	
	+	+	+			+		3	
	+	+	+			0.5		3	
	+	+	+			1		2 to 3	

+ indicates standard concentration used, numbers indicate other concentration used.

Passaged cultures are indicated by thicker border in grading column and grading of p1 cultures.

5 [0085] Table 3 indicates the titrations that were analyzed. The base media comprised 1:1 DMEM/F12, 2 mM L-glutamine, 1x non-essential amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM 2-Mercaptoethanol, 8 ng/ml FGF2, and 10  $\mu$ g/ml transferrin. The components that were varied were LongR<sup>3</sup>-IGF1 (50-500

ng/ml), Activin A (0.1-10 ng/ml), BMP4 (0.05-10 ng/ml), TGF $\beta$  (0.1-20 ng/ml), Nodal (1-50 ng/ml), or BSA (0.02-1%). BSA is successfully replaced with human serum albumin (HSA).

5 [0086] Low-dose activin (approximately 0.1-10 ng/ml) allowed for proliferation of undifferentiated hESCs, which were graded either 3 to 4, or 4. The cultures grown in the different conditions looked similar, and neural rosette differentiation appeared lower than a control (no activin) culture (data not shown). 1 ng/ml Activin was selected as the standard concentration for later experiments, and such cultures were successfully passaged.

10 [0087] Undifferentiated cells successfully proliferated in approximately 0.05 or 0.1 ng/ml BMP4, but were not successfully passaged. Higher concentrations of BMP4, 1-10 ng/ml, caused differentiation.

15 [0088] LongR<sup>3</sup>-IGF1 was titrated from approximately 50 to 500 ng/ml. Cultures grown in all the conditions containing LongR<sup>3</sup>-IGF1 were graded at 4. 100 ng/ml LongR<sup>3</sup>-IGF1 was selected for use in later experiments. Some of these cultures were selected for passaging and were successfully passaged.

20 [0089] TGF- $\beta$  was titrated from approximately 0.1 – 20 ng/ml. Cultures grown in all the conditions containing TGF- $\beta$  were graded at 3 to 4. Overall, there were large undifferentiated regions within colonies, but cells in the colonies were not as tightly packed as expected. Some of these cultures were passaged, but the cells differentiated.

[0090] Nodal was titrated from approximately 1 – 50 ng/ml. Cultures containing 2.5 or 5 ng/ml nodal were given a grading of 4, with higher and lower concentrations receiving a lower grading. Some of these cultures were passaged, but the cells differentiated.

25 [0091] BSA was titrated from approximately 0.02 – 1%. The conditions for this experiment were not optimal, such that the cultures exhibited higher differentiation than typical and received a lower grading overall than in other experiments. However, it was apparent that 0.1-0.5% was a suitable concentration range for BSA, with cultures treated with a lower concentration exhibiting cell death, and cultures with a 30 higher concentration exhibiting increased differentiation.

**Example 4***Passaging of cells grown in defined media*

[0092] The ability to passage and expand cells whilst maintaining pluripotency 5 is a key step in the development of defined media conditions for hESCs. During the experiments described in Examples 1, 2, and 3, Applicants attempted to passage cultures that exhibited substantial proliferation of undifferentiated cells (grades 3 to 5).

[0093] Cultures were successfully passaged using collagenase to disaggregate 10 the colonies, generating p1 cultures (Table 3), however, manual passaging methods were less successful. These cells were passaged to the same media that each of the p0 cultures was in, and were graded at days 4-5 after passaging, with 8-10 total days in the defined media. Cultures containing 0.05 or 0.1 ng/ml BMP4 exhibited colonies of differentiated cells (grade 2). Cultures containing TGF $\beta$  were primarily differentiated 15 (grades 1 or 2). A culture with 5 ng/ml nodal contained some undifferentiated cells (grade 3), but at lower concentrations the cultures were primarily differentiated (grades 2, or 2 to 3).

[0094] P1 cultures grown in 1 ng/ml activin and 100, 200, or 300 ng/ml 20 LongR $^3$ -IGF1 all contained some colonies of undifferentiated cells, and were graded at 3 to 4. A culture containing 1 ng/ml activin and 500 ng/ml LongR $^3$ -IGF1 contained a higher level of differentiation and was grade 3.

[0095] The morphologies of p1 colonies grown in either 8 ng/ml FGF2 and 100 25 ng/ml LongR $^3$ -IGF1, or in 8 ng/ml FGF2, LongR $^3$ -IGF1 and 1 ng/ml Activin A are shown in Figures 3A-D.

[0096] To examine benefit of plating hESCs on alternate extracellular matrices, 30 p0 cultures were grown in 8 ng/ml FGF2, 100 ng/ml LongR $^3$ -IGF1 and 1 ng/ml Activin A on dishes coated with human fibronectin (Invitrogen). Tissue culture dishes were coated with 10  $\mu$  g/ml human plasma fibronectin (Invitrogen, #33016-015), diluted in tissue grade water, for 2-3 hours at room temperature. The dishes were washed with tissue culture grade water before use. To prepare these p0 cultures, parental BG01v cells were passaged to p1 cultures with Accutase (Innovative Cell Technologies) according to the manufacturer's instructions. Accutase comprised 1x accutase enzymes in PBS, containing 0.5 mM EDTA. These p0 cultures were graded at 4-5, and were successfully passaged to p1 and subsequently p2 cultures with Accutase passaging. The p1 and p2 cultures were grown on human fibronectin in the presence of

defined medium comprising 1:1 DMEM/F12, 2 mM L-glutamine, 1x non-essential amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol, 8 ng/ml FGF2, 10  $\mu$ g/ml transferrin, 0.2% BSA, 100 or 200 ng/ml LongR<sup>3</sup>-IGF1, and 1 ng/ml Activin A. These cultures were grade 4 at p1 and grade 3 at p2. The p2 cultures 5 consisted almost entirely of morphologically undifferentiated cells in colonies, but appeared to have slower growth and smaller colonies than observed in p0 cultures, which accounts for the lower grading. If the slower growth rate was not considered, these p2 cultures were grade 4-5.

[0097] 0.01% defined lipids (Invitrogen), 50  $\mu$ g/ml ascorbic acid, and 0.0017 10  $\mu$ g/ml sodium selenite were added to the p2 culture containing 100  $\mu$ g/ml LongR<sup>3</sup>-IGF1, two days after plating, to assess if this caused obvious improvements in the growth rate. No obvious effect was observed after 2 days, and cultures were harvested for analysis 4 days after plating at p2.

[0098] RNA was prepared from a p2 culture containing 100  $\mu$ g/ml LongR<sup>3</sup>-IGF1 and was analyzed by RT-PCR as described previously (Brimble *et al.*, 2004 Stem Cells Dev. 13:585-596). These p2 cultures expressed multiple markers of pluripotency, including ABCG2, DPPA5, REX1, UTF1, NANOG, OCT4, SOX2, CRIPTO, and TERT (Figure 4A).

[0099] A p2 culture containing 200  $\mu$ g/ml LongR<sup>3</sup>-IGF1 was fixed in 4% 20 paraformaldehyde (PFA) (Fisher Scientific, Hampton, NH) and 4% sucrose (Sigma) in 1x PBS. The samples was blocked with 3% goat serum (Invitrogen), 1% polyvinyl pyrrolidone (Sigma), and 0.3% Triton X-100 (Sigma) in 1x PBS (block buffer) and then incubated with primary antibodies diluted in block buffer for 1 hour at room 25 temperature. The primary antibodies were mouse anti-SSEA-4 (Chemicon) and rabbit anti-OCT4 (Santa Cruz). The sample was then washed and incubated for 1 hour in secondary antibodies diluted 1:1,000 in block buffer, followed by washing. The secondary antibodies were goat anti-rabbit antibody conjugated with alexa594 (red), and goat anti-mouse antibody conjugated with Alexa-488 (green), (Molecular Probes). Nuclei were stained with 5 ng/ml 4',6'-diamidino-2-phenylindole (DAPI; Sigma; Figure 30 4B). Images of SSEA-4 and OCT4 expression were captured, and these p2 colonies exhibited uniform expression of both markers (Figures 4C and D). Alkaline phosphatase expression in the p2 culture was subsequently detected using NBT/BCIP

(Roche # 1697471) according to the manufacturer's instructions. The colonies were also uniformly positive for alkaline phosphatase activity (Figure 4E).

[00100] P2 cultures are successfully passaged to further cultures in the presence of defined media, without losing the characteristics of pluripotent cells.

5

### Example 5

*Different extracellular matrices can be used with the defined medium*

[00101] The growth of HESCs in defined medium on different extracellular matrices was compared. Cells were easily grown on dishes coated with matrigel, 10 fibronectin, and a fibronectin/laminin mixture. However, cells were not easily grown on dishes coated with laminin, polyornithine, or human albumin. In addition, data suggested that there is more neural differentiation in the p1 cultures when cells were grown on matrigel than when cells were grown on fibronectin.

15

### Example 6

*Use of a lipid source in the defined media*

[00102] BG01v cells were cultured as described above, with minor changes. BG01v cells were plated to 6-well dishes that had been coated with fibronectin. Tissue culture dishes were coated with 10 µg/ml human plasma fibronectin (Invitrogen, 20 #33016-015), diluted in tissue grade water, for 2-3 hours at room temperature. The dishes were washed with tissue culture grade water before use.

[00103] The cells were cultured directly in the defined media conditions, or in 20% KSR MEF-CM overnight to promote adherence of colony pieces before switching to defined media. The defined media comprised approximately 1:1 DMEM/F12, 25 2 mM L-glutamine, 1x non-essential amino acids, 0.5 U/ml penicillin, 0.5 U/ml streptomycin, 0.1 mM β-Mercaptoethanol, 10 µg/ml transferrin, 8 ng/ml FGF2, 50 ng/ml LongR<sup>3</sup>-IGF1 and 1 ng/ml Activin A. The concentrations of different lipid sources were titrated within the defined media, in order to identify the optimal concentrations of lipids. Ex-Cyte lipids and Invitrogen defined lipids were titrated 30 ranging from approximately 0.01% to approximately 1%.

[00104] BG01v cells expanded effectively in defined media containing lipids, forming large colonies of undifferentiated cells, with low levels of differentiation. These cultures were grades 4 to 5. The p0 cultures were successfully passaged using

accutase to p1 cultures as described above. The morphological gradings of the p1 cultures are shown below in Tables 4-5.

5 [00105] Tables 4 and 5 indicate the titrations analyzed, and the graded morphology of the p1 cultures. The addition of lipids results in changes in the morphology of the colonies: they are more three-dimensional, although the cells still grow as a single layer within the colony. The colonies are thicker in the presence of the lipids, and refract the light more than colonies cultured without lipids.

Table 4

10

Ex-Cyte lipids	Grade of p1 culture	Comments
0.01%	3-4	Predominantly undifferentiated cells
0.05%	3-4	Predominantly undifferentiated cells
0.1%	3	Mix of undifferentiated and differentiating cells
0.2%	2-3	Predominantly differentiated cells
0.5%	1-2	Predominantly differentiated cells
1%	1	Predominantly differentiated cells

Table 5

Invitrogen Defined lipids	Grade of p1 culture	Comments
0.01%	3-4	The undifferentiated colonies are larger than with Ex-Cyte lipids, but there are some differentiating cells between the colonies that are not observed with the Ex-Cyte lipids, or without lipids
0.05%	3-4	As above
0.1%	3-4	As above
0.2%	3	The differentiated cells are more prevalent than in the lower concentrations of lipids
0.5%	3	As above
1%	3	As above

15 [00106] P1 cultures are successfully passaged to further cultures in the presence of defined media containing lipids at a concentration of approximately 0.0001% to approximately 1%, without losing the characteristics of pluripotent cells.

20

## CLAIMS

## WE CLAIM:

- 5 1. A composition consisting essentially of a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free.
- 10 2. The composition of Claim 1, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.
- 15 3. The composition of Claim 1, wherein the member of the TGF- $\beta$  family is Activin A.
4. The composition of Claim 1, wherein the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor.
5. The composition of Claim 1, wherein the activator of IGF-1R is insulin.
6. The composition of Claim 1, wherein the activator of IGF-1R is an insulin-like growth factor.
7. The composition of Claim 6, wherein the insulin-like growth factor is IGF-1 or IGF-2.
- 20 8. The composition of Claim 7, wherein the IGF-1 is LongR<sup>3</sup>IGF-1.
9. The composition of Claim 1, wherein the activator of an FGF receptor is FGF2.
10. The composition of Claim 1, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.
- 25 11. A composition for culturing a pluripotent mammalian embryonic stem cell, comprising:  
an extracellular matrix, and

a medium consisting essentially of a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the medium is essentially serum free,

5       wherein a pluripotent mammalian stem cell remains undifferentiated for greater than approximately 1 month in culture.

12.     The composition of Claim 11, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and  
10     BMP4.

13.     The composition of Claim 11, wherein the member of the TGF- $\beta$  family is Activin A.

14.     The composition of Claim 11, wherein the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor.

15     15.     The composition of Claim 11, wherein the activator of IGF-1R is insulin.

16.     The composition of Claim 11, wherein the activator of IGF-1R is an insulin-like growth factor.

17.     The composition of Claim 16, wherein the insulin-like growth factor is IGF-1 or IGF-2.

20     18.     The composition of Claim 17, wherein the IGF-1 is LongR<sup>3</sup>IGF-1.

19.     The composition of Claim 11, wherein the activator of an FGF receptor is FGF2.

20.     The composition of Claim 11, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.

25     21.     The composition of Claim 11, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.

22. The composition of Claim 21, wherein the mammalian embryonic stem cell is a human embryonic stem cell.

23. The composition of Claim 21, wherein the medium is a non-conditioned medium.

5 24. A composition comprising

10 a pluripotent mammalian embryonic stem cell proliferating on an extracellular matrix in the presence of a defined medium, wherein the cell composition is essentially free of feeder cells and essentially serum free, and wherein the defined medium consists essentially of a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family.

25. The composition of Claim 24, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.

15 26. The composition of Claim 24, wherein the member of the TGF- $\beta$  family is Activin A.

27. The composition of Claim 24, wherein the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor.

28. The composition of Claim 24, wherein the activator of IGF-1R is insulin.

20 29. The composition of Claim 24, wherein the activator of IGF-1R is an insulin-like growth factor.

30. The composition of Claim 29, wherein the insulin-like growth factor is IGF-1 or IGF-2.

31. The composition of Claim 30, wherein the IGF-1 is LongR<sup>3</sup>IGF-1.

25 32. The composition of Claim 24, wherein the activator of an FGF receptor is FGF2.

33. The composition of Claim 24, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.

34. The composition of Claim 24, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.

5 35. The composition of Claim 24, wherein the mammalian embryonic stem cell is a human embryonic stem cell.

36. The composition of Claim 24, wherein the medium is a non-conditioned medium.

10 37. The composition of Claim 24, wherein the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than one month in culture.

38. A method of culturing a pluripotent mammalian embryonic stem cell comprising  
(a) providing a pluripotent mammalian embryonic stem cell;  
(b) plating the cell on an extracellular matrix; and  
15 (c) contacting the cell with a defined medium that is essentially serum free consisting essentially of a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family,  
wherein the stem cell proliferates in culture and remains undifferentiated essentially in  
20 the absence of serum or serum replacement in the medium.

39. The method of Claim 38, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.

40. The method of Claim 38, wherein the member of the TGF- $\beta$  family is Activin  
25 A.

41. The method of Claim 38, wherein the activator of IGF-1R is selected from the group consisting of insulin and an insulin-like growth factor.

42. The method of Claim 38, wherein the activator of IGF-1R is insulin.

43. The method of Claim 38, wherein the activator of IGF-1R is an insulin-like growth factor.
44. The method of Claim 43, wherein the insulin-like growth factor is IGF-1 or IGF-2.
- 5 45. The method of Claim 44, wherein the IGF-1 is LongR<sup>3</sup>IGF-1.
46. The method of Claim 38, wherein the activator of an FGF receptor is FGF2.
47. The method of Claim 38, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.
- 10 48. The method of Claim 38, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.
49. The method of Claim 38, wherein the mammalian embryonic stem cell is a human embryonic stem cell.
50. The method of Claim 38, wherein the medium is a non-conditioned medium.
- 15 51. The method of Claim 38, wherein the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than one month in culture.
52. The method of Claim 38, wherein the contact with the defined medium is in the absence of a feeder layer.
- 20 53. The method of Claim 38, wherein the pluripotent mammalian embryonic stem cell is cultured on feeder cells prior to plating on the extracellular matrix.
54. A composition comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free.
- 25 55. The composition of Claim 54, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.

56. The composition of Claim 54, wherein the activator of IGF-1R is selected from the group consisting of insulin, IGF-1, and IGF-2.
57. The composition of Claim 54, wherein the activator of an FGF receptor is FGF2.
58. The composition of Claim 54, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.
59. A composition for culturing a pluripotent mammalian embryonic stem cell, comprising:
  - an extracellular matrix, and
  - a medium comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the medium is essentially serum free, wherein a pluripotent mammalian stem cell remains undifferentiated for greater than approximately 1 month in culture.
- 15 60. The composition of Claim 59, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.
61. The composition of Claim 59, wherein the activator of IGF-1R is selected from the group consisting of insulin, IGF-1, and IGF-2.
- 20 62. The composition of Claim 59, wherein the activator of an FGF receptor is FGF2.
63. The composition of Claim 59, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.
- 25 64. The composition of Claim 59, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.
65. The composition of Claim 59, wherein the mammalian embryonic stem cell is a human embryonic stem cell.

66. The composition of Claim 59, wherein the medium is a non-conditioned medium.

67. A composition comprising

5 a pluripotent mammalian embryonic stem cell proliferating on an extracellular matrix in the presence of a defined medium, wherein the cell composition is essentially free of feeder cells and essentially serum free, and wherein the defined medium comprises basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family.

10 68. The composition of Claim 67, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.

69. The composition of Claim 67, wherein the activator of IGF-1R is selected from the group consisting of insulin, IGF-1, and IGF-2.

15 70. The composition of Claim 67, wherein the activator of an FGF receptor is FGF2.

71. The composition of Claim 67, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.

20 72. The composition of Claim 67, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.

73. The composition of Claim 67, wherein the mammalian embryonic stem cell is a human embryonic stem cell.

25 74. The composition of Claim 67, wherein the medium is a non-conditioned medium.

75. The composition of Claim 67, wherein the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than one month in culture.

76. A method of culturing a pluripotent mammalian embryonic stem cell comprising  
(a) providing a pluripotent mammalian embryonic stem cell;  
(b) plating the cell on an extracellular matrix; and  
(c) contacting the cell with a defined medium that is essentially serum free  
5 comprising a basal salt nutrient solution, an activator of IGF-1R, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family,

wherein the stem cell proliferates in culture and remains undifferentiated essentially in the absence of serum or serum replacement in the medium.

10 77. The method of Claim 76, wherein the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, and BMP4.

78. The method of Claim 76, wherein the activator of IGF-1R is selected from the group consisting of insulin, IGF-1, and IGF-2.

15 79. The method of Claim 76, wherein the activator of an FGF receptor is FGF2.

80. The method of Claim 76, wherein the serum albumin is selected from the group consisting of bovine serum albumin and human serum albumin.

81. The method of Claim 76, wherein the pluripotent mammalian embryonic stem cell is selected from the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.

20 82. The method of Claim 76, wherein the mammalian embryonic stem cell is a human embryonic stem cell.

83. The method of Claim 76, wherein the medium is a non-conditioned medium.

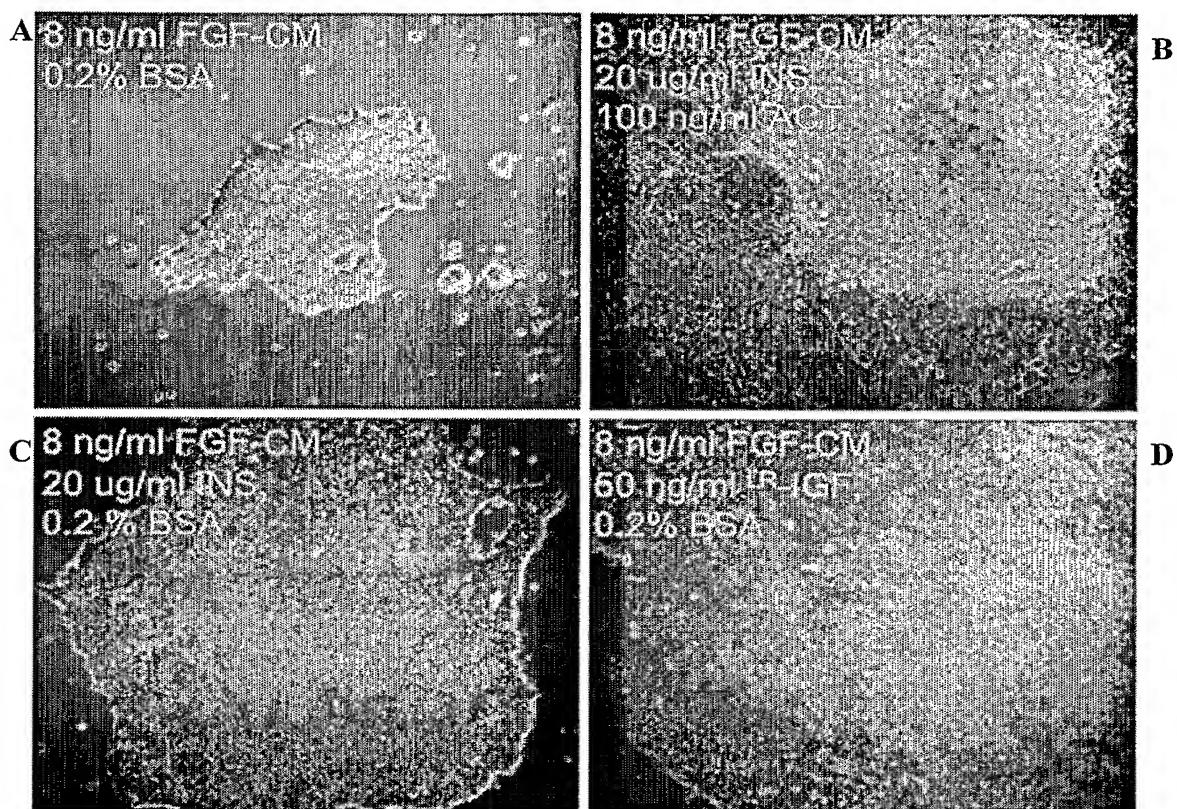
25 84. The method of Claim 76, wherein the pluripotent mammalian embryonic stem cell proliferates and remains undifferentiated for greater than one month in culture.

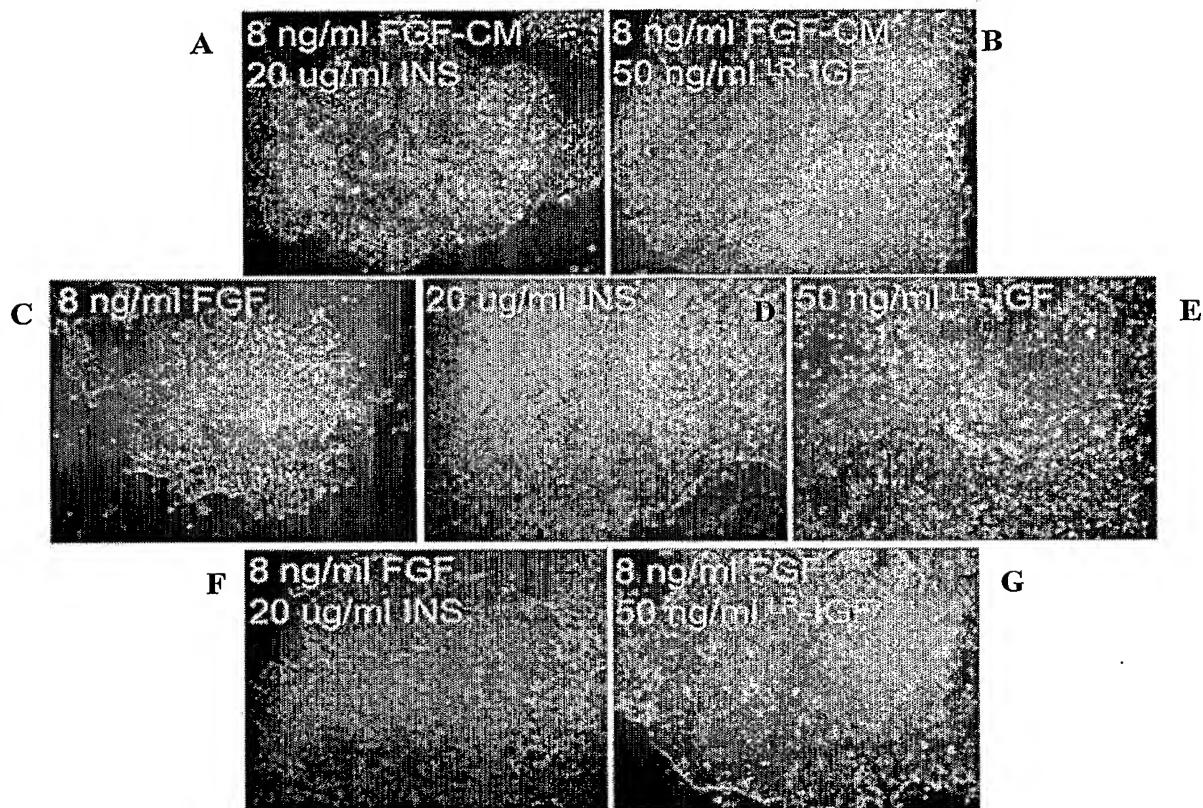
85. The method of Claim 76, wherein the contact with the defined medium is in the absence of a feeder layer.

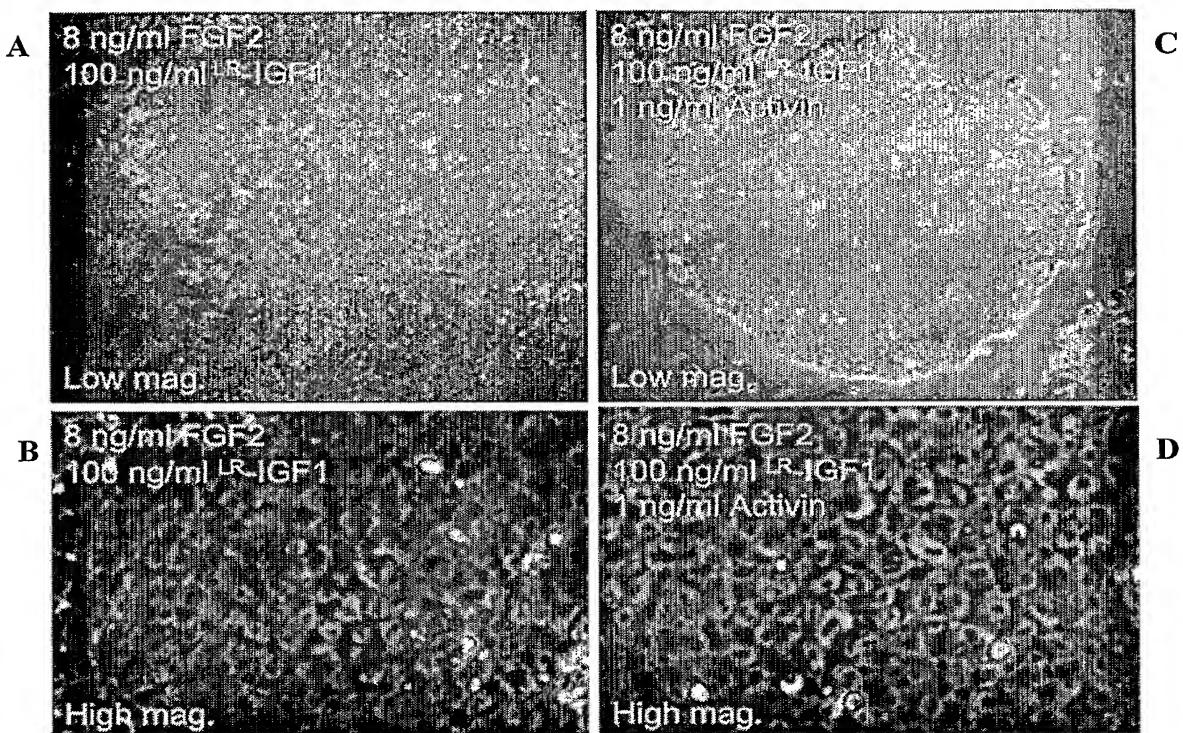
86. The method of Claim 76, wherein the pluripotent mammalian embryonic stem cell is cultured on feeder cells prior to plating on the extracellular matrix.

87. A composition consisting essentially of a basal salt nutrient solution, an activator of the PI3K pathway, serum albumin, an activator of an FGF receptor, transferrin, and optionally, a member of the TGF- $\beta$  family, wherein the composition is essentially serum free.

5 88. The composition of Claim 87, wherein the activator of the PI3K pathway is an activator of IGF-1R and wherein the composition further comprises a second activator of the PI3K pathway.

**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**